Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis

Thrombospondin Cooperates with CD36 and the Vitronectin Receptor in Macrophage Recognition of Neutrophils Undergoing Apoptosis

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Abstract

We have investigated the cell surface recognition mechanisms used by human monocyte-derived macrophages (Mϕ) in phagocytosis of intact aging human neutrophils (PMNs) undergoing apoptosis. This study shows that the adhesive protein thrombospondin (TSP) was present in the interaction, both associated with the Mϕ surface and in solution at a mean concentration of 0.59 μg/ml. The interaction was inhibited by treatment of Mϕ (but not aged PMN) with cycloheximide, but could be "rescued" by replenishment with exogenous TSP. Under control conditions, Mϕ recognition of aged PMNs was specifically potentiated by purified platelet TSP at 5 μg/ml, present either in the interaction or if preincubated with either cell type, suggesting that TSP might act as a "molecular bridge" between the two cell types. In support, both aged PMN and Mϕ were found to adhere to TSP, and phagocytosis of aged PMN was specifically inhibited by (*a*) excess soluble TSP; (*b*) antibodies to TSP that also inhibit TSP-mediated adhesion to aged PMN; and (*c*) down-regulation of Mϕ receptors for TSP by plating Mϕ on TSP-coated surfaces. Furthermore, inhibition with mAbs/Arg-Gly-Asp-Ser peptide of the candidate Mϕ receptors for TSP, CD36, and $\alpha_v\beta_3$ exerted synergistic effects on both Mϕ recognition of aged PMN and Mϕ adhesion to TSP, indicating that "two point" adhesion of TSP to these Mϕ structures is involved in phagocytosis of aged PMN. Our findings indicate newly defined roles for TSP and CD36 in phagocytic clearance of senescent neutrophils, which may limit inflammatory tissue injury and promote resolution. (*J. Clin. Invest.* 1992. 90:1513–1522.) Key words: adhesion • integrins • resolution • inflammation • leukocytes

Introduction

In inflammation, neutrophils and their contents can injure tissue and may generate chemotactic factors with potential to amplify leukocyte infiltration (1, 2, 3). Safe removal of neutrophils from the perturbed site is, therefore, a prerequisite of resolution of inflammation. Uptake of intact senescent neutrophils by macrophages at resolving inflamed sites was recognized many years ago (4), and, for example, has been shown to be the dominant mechanism of neutrophil clearance from the experimentally inflamed peritoneum (5, 6). However, only recently have the mechanisms involved in this cellular interaction come under study. Building on earlier work (7), we showed that human neutrophils isolated from blood or inflamed sites and aged for ~ 24 h in culture undergo morphological and biochemical changes typical of programmed cell death (apoptosis), a process characterized by evidence of endogenous endonuclease activation (8–11). Apoptosis in aging neutrophils determined recognition and uptake of the intact senescent cell by human monocyte-derived macrophages and human macrophages isolated from inflamed sites in vivo. As apoptotic neutrophils retained their membrane integrity and did not release toxic contents, these processes appear to represent a mechanism for disposal of intact senescent neutrophils in a manner likely to limit their toxic potential (8). In the present study, we examine the cell surface mechanisms used by macrophages in recognition of apoptotic neutrophils.

Previously, we found that the macrophage $\alpha_v\beta_3$ integrin, or vitronectin receptor (VnR),[1] has a role in recognition of aged neutrophils (12). The interaction depended on the divalent cations Ca^{++} and Mg^{++}, and was specifically inhibited by (*a*) the tetrapeptide Arg-Gly-Asp-Ser (RGDS) at 1 mM, but not the control peptide Arg-Gly-Glu-Ser (RGES); (*b*) by soluble and substrate-bound fibronectin (Fn) and vitronectin (Vn), but not fibrinogen or type IV collagen, which also bear the RGD recognition sequence; and (*c*) by monoclonal antibodies to both subunits of the $\alpha_v\beta_3$ integrin, which is expressed by the Mϕ not the apoptotic neutrophil (12–14). It was concluded that the VnR played a direct role in the recognition mechanism, a new function for this receptor in addition to its known roles in cell anchorage to matrix proteins (13). However, Mϕ recognition of aged neutrophils was also directly modulated by pH and specifically inhibited by millimolar concentrations of aminosugars and basic aminoacids in a charge-dependent fashion (15), and such observations were not explained by the molecular interactions of the Mϕ VnR.

Cationic amino sugars and basic amino acids also inhibit the trypsin-sensitive "lectin-like" property displayed by activated platelets in agglutination of fixed platelets and fixed trypsinized erythrocytes (16). Subsequently, the activated platelet "lectin" was shown to be thrombospondin (TSP) (17). TSP is a "multifunctional" trimeric adhesive molecule of ~ 450 kD capable of binding to a wide range of macromolecules and many cell types (18–22). TSP has been implicated as a "molec-

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1. *Abbreviations used in this paper:* EIgG, ox red erythrocytes opsonized with rabbit IgG; Fn, fibronectin; Mϕ, monocyte-derived macrophages; RGDS, tetrapeptide Arg-Gly-Asp-Ser; RGES, control peptide Arg-Gly-Glu-Ser; TSP, thrombospondin; Vn, vitronectin; VnR, $\alpha_v\beta_3$ vitronectin receptor.

ular bridge" mediating adhesive interactions between activated platelets and other cells, including erythrocytes and monocyte/macrophages (23–25). However, the mechanisms by which TSP binds to cells are incompletely understood and potentially complex, since there is evidence that a number of cell surface molecules may be involved, including sulfatides, proteoglycans and proteins, including $\alpha_v\beta_3$ and the 88-kD monomer GPIV/CD36 (23, 25–28). Although first isolated from the α-granule of the platelet, TSP has been found to be synthesized and secreted by a wide range of cell types in culture (20–22), including both macrophages and neutrophils (29, 30), and there is evidence that each cell type may bear receptors for TSP (28, 31–34). Indeed, macrophages may have TSP associated with their surfaces (33, 34). Furthermore, TSP has been shown to be a transient component of the inflammatory extracellular matrix of healing wounds present at the time in which neutrophils are removed from the tissue (35), and has also been demonstrated in lavage fluid from inflamed lungs (36). Consequently, in the present study, we sought evidence that TSP and TSP-binding structures were involved in Mϕ recognition of aged neutrophils. Our data indicate that thrombospondin can act as a "molecular bridge" between the aged neutrophil and the Mϕ, where the molecule appears to bind to both GPIV/CD36 and the $\alpha_v\beta_3$ integrin, indicating hitherto unrecognized functions for both TSP and GPIV/CD36.

Methods

Materials

All chemicals were from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated; culture media (HBSS, Iscove's DME) and supplements (100 U/liter of penicillin and streptomycin) were from Gibco Laboratories (Grand Island, NY); and sterile tissue culture plasticware were from Falcon Plastics (Cockeysville, MD).

Peptides and proteins

The tetrapeptides RGDS and RGES were obtained from Peninsula Laboratories, Inc., (Belmont, CA), human albumin (fraction V) was from Sigma and fibronectin from Calbiochem Corp. (San Diego, CA). TSP was purified from thrombin-stimulated human platelets by heparin-Sepharose affinity chromatography followed by "sizing" on a 4B-Sepharose column by standard methods (18). Particular care was taken to use only those fractions in which the protein ran as a single band on reducing SDS-PAGE assessed by silver staining and immunoblotting with a mAb specific for TSP (TSP-B7; Sigma). Contamination with Vn was quantified at $< 0.1\%$ by specific ELISA (data courtesy of Dr. W. Bennett). Proteins and peptides were dialyzed in HBSS before use.

Monoclonal antibodies

Dr. V. Dixit kindly provided a panel of IgG_1 mAbs specific for TSP, namely C6.7, A6.1, A2.5, and D4.6, each of which has been shown to inhibit various TSP-related phenomena (37–39); Drs R. Nachman and R. Silverstein provided the TSP-specific mAb 11.2 (24). CD36 mAbs were SMϕ, an IgM (40, 41) and C1Meg1, an IgG_1 (40) (a gift from Dr. G. Pilkington). The $\alpha_v\beta_3$ mAb was 23C6 (42) (a gift from Dr. M. Horton). Control mAbs were P3 (43) an irrelevant IgG_1 (a gift from Dr. P. Morganelli), P112 (44) an IgM that recognizes the GPIIbIIIa complex, 28 (40, 44) an IgM recognizing CD15, and OX7 (Serotec, Banbury, Oxon, U.K.), an IgG_1 that recognizes Thy1.1. Antibodies were used as purified immunoglobulin (23C6, 11.2,P3) or ascites (C6.7, A2.5, A6.1, D4.6, SMϕ, C1Meg1, 23C6, P112, OX7) diluted in HBSS as described.

Cells

Neutrophils (> 98% pure May-Giemsa) were isolated from fresh, citrated normal human blood by dextran sedimentation and plasma-Percoll (Pharmacia Fine Chemicals, Piscataway, NJ), aged in tissue culture for ~ 28 h in Iscove's DME with 10% autologous platelet-rich, plasma-derived serum, and apoptosis verified by oil-immersion light microscopy of May-Giemsa stained cytopreps, exactly as previously described (8, 15). Human monocyte-derived macrophages (Mϕ) were prepared by standard methods from adherent PBMC by culture for 5–7 d in 24-well plates in Iscove's medium with 10% autologous serum as described (45, 8). No contaminating platelets attached to Mϕ could be detected either by careful microscopic inspection or by immunofluorescence using mAb P112 specific for platelet GPIIbIIIa. For demonstration of cell surface TSP by flow cytometry (see below), monocytes were purified by elutriation (46) and matured in nonadherent culture in teflon-lined vessels for 5–7 d. Ox erythrocytes, opsonized with polyclonal rabbit anti-ox IgG (gift from Dr. D. Grennan) were prepared as described (15).

Assay and localization of TSP

TSP in solution in medium was assayed by ELISA exactly as described (34), using rabbit polyclonal immunoglobulin specific for TSP (1:5,000 in PBS; a gift from Dr. N. Hunter); this assay was sensitive to a lower limit of 50 ng/ml. TSP was sought on the surface of cultured Mϕ and neutrophils by immunofluorescence flow cytometry, using a standard protocol of labeling and three washes at the end of each step, as described (12). Binding of C6.7 (1:100 dilution in PBS/0.1% BSA) was compared with an identical dilution of OX7 ascites as a control, detected using FITC-conjugated Fab'_2 sheep anti-mouse immunoglobulin antibody (1:30; Sigma). Aged neutrophils were studied by the same techniques.

Interaction assays

This microscopically quantified phagocytic assay has been described and illustrated in detail before (8, 15). Briefly, aged neutrophils were washed once in HBSS and suspended in HBSS. 2.5×10^6 aged PMN in 300 μl HBSS were added to each washed well of Mϕ and incubated at 37°C in a 5% CO_2 incubator at pH 7.4. In view of our previous findings of the importance of pH (15), particular care was taken to ensure that the pH of HBSS was adjusted and maintained correctly. At the end of the interaction period, the wells were washed in saline at 4°C, fixed with 2% glutaraldehyde in PBS, stained for myeloperoxidase and then the proportion of Mϕ ingesting neutrophils counted by inverted light microscopy, exactly as described (8, 15). In keeping with our previous description of this assay, the proportion of Mϕ recognizing aged neutrophils varied between donors, so to compare data between experiments in which Mϕ from different donors were used, data are presented as percentage of the mean of control in each of the relevant experiments, as described (15). However, the absolute percentage of Mϕ recognizing aged neutrophils in the relevant series of experiments is also given in the figure legend. Uptake of opsonized ox erythrocytes (EIgG) was determined by similar means, as described (15). In all experiments, > 95% of Mϕ took up EIgG.

Effects of proteins in solution. TSP or human albumin were included in the interaction medium at the desired concentration in HBSS and interactions performed in HBSS alone served as control. In preincubation experiments, proteins at 5 μg/ml were incubated with washed aged neutrophils or Mϕ at 37°C for 15 min (HBSS alone being used in "control" experiments), washed and then incubated with untreated Mϕ or neutrophils as appropriate under the standard conditions of the assay.

Effects of monoclonal antibodies. These were determined as previously described (12, 15). Briefly, adherent Mϕ cultured in 24-well plates were washed, 300 μl of mAb at desired concentration in HBSS (or HBSS alone as a control) was added to each well. The plates were incubated for 15 min at 4°C, followed by addition of 2.5×10^6 washed aged neutrophils (or EIgG) in 100 μl of warm HBSS and then interac-

tion for 30 min under standard conditions. In no experiment did mAbs affect cell viability as assessed by trypan blue exclusion. In one series of experiments designed to determine the cellular localization of the effect of mAb SMϕ either aged neutrophils (at 2.5×10^6/ml) or adherent Mϕ were preincubated with mAb SMϕ (1:25 dilution) for 30 min at 4°C, before washing twice in HBSS and interaction with untreated cells of the opposite type under standard conditions. These effects were compared with those of presence of mAb at the same concentration in the interaction medium, without the usual 15-min Mϕ preincubation.

It should be noted that in previous work, a large number of murine mAbs of varying isotype have been examined in this assay in the form of intact Ig (either purified or as hybridoma ascites) (12, 15). Except for mAbs to $\alpha_v\beta_3$, none has inhibited, including mAbs to Mϕ and neutrophil surface structures, indicating that nonspecific "steric" effects are unlikely to be the reason for the blocking effects of mAb in this system. Furthermore, no mAb (including those used in this study) have promoted Mϕ phagocytosis of freshly isolated neutrophils in a manner suggesting that murine mAbs might opsonize neutrophils for Mϕ, thus complicating the interpretation of these data.

Effects of plating Mϕ on tissue culture surfaces treated with proteins. TSP was adsorbed to the bottom of tissue culture-treated wells by standard methods (47–49, 27). 50 μl of a solution of TSP or human albumin at 80 μg/ml in HBSS was incubated in each well of a 96-well flat-bottomed Falcon tissue culture plate for 2 h at room temperature and washed twice in HBSS and "control" wells were incubated with HBSS alone. Mature Mϕ were "mobilized" from adherent culture by methods shown by immunofluorescence flow cytometry to "strip" them of surface-associated TSP (data not shown) to facilitate modulation of receptors by substrate-bound proteins. Thus Mϕ were incubated with 5 mM EDTA in HBSS with no Ca^{++} or Mg^{++} for 15 min at 4°C, detached from the plate by pipetting, washed twice in warm HBSS, suspended at 5×10^5/ml and 50 μl of suspension added to each well. The cells, which formed a "subconfluent" monolayer, underwent receptor modulation (or not) during incubation at 37°C for 30 min. The medium was then aspirated and replaced with 100 μl of a suspension of aged neutrophils in HBSS at 2.5×10^6/ml, or EIgG at 5×10^6/ml for a 30-min interaction. Counting of Mϕ by inverted microscopy showed that < 10% of Mϕ originally added to the well were lost during these steps, indicating that adherent Mϕ were not a subpopulation.

Assay of adhesion of aged neutrophils to latex beads

In preliminary experiments, apoptotic PMN did not spread on tissue culture-treated surfaces or hemocytometer glass, remaining spherical and resisting mechanical displacement only weakly (Whyte, M.K.B., J.S. Savill, and C. Haslett; unpublished data). Consequently, we sought adhesion to protein-coated 0.995-μm latex beads (LB11; Sigma) by minor modifications of standard methods (50, 51), since this does not require neutrophil spreading. A 300-μl aliquot of a 10% stock suspension of latex beads was added to a 1.5-ml polythene Eppendorf tube, pelleted and washed three times in 0.9% saline, and then incubated with either TSP or human Fn (Calbiochem) at 100 μg/ml in 0.9% saline with Tris HCl 20 mM, pH 7.4, for 10 min at room temperature. The bead suspension was then sonicated with a probe sonicator (60 W) to break up microclumps, centrifuged and resuspended in 10 mg/ml human albumin in HBSS, then incubated for a further 10 min at room temperature. Beads were pelleted and washed three times in HBSS before resuspension in 500 μl HBSS; i.e., a 6% suspension. TSP coating was confirmed by positive indirect immunofluorescence with mAb 11.2 at 2.5 μg/ml and negative staining with irrelevant mAb P3 at the same concentration. Aged neutrophils were washed and resuspended at 50×10^6/ml in HBSS, and 225 μl were added to each test tube (Falcon LP3 polythene tubes). In some experiments TSP mAbs C6.7, A2.5, A6.1, and D4.6 were included in the medium at a dilution of 1 in 25 of ascites. 25 ml of freshly prepared 6% beads was added to the cell suspension to achieve a final bead concentration of 0.6%, and the tube was shaken in a 37°C water bath at 110 beats/min for 15 min, supplemented by manual agitation at 7 min. In preliminary experiments under these conditions, TSP mAbs at the concentration described above did not aggregate either beads or aged neutrophils alone, indicating that inhibitory effects on bead binding were unlikely to be by such "artefactual" mechanisms that might have reduced the area of interface between beads and cells. The cells were fixed by addition of 250 μl of 2% glutaraldehyde in PBS for 10 min, and then cells separated from unbound beads (which are of lower density) by four consecutive centrifugations at 200 *g* for 4 min with washing in 0.9% saline, before finally suspending beads in 250 μl 0.9% saline and placing 50 μl on a microscope slide. The cells were inspected by oil immersion light microscopy after placing a cover slip on the drop. 500 neutrophils were examined to determine the percentage of cells binding beads, and then the number of beads on each of 100 cells with beads were counted.

Assay of Mϕ adhesion

Established methods (47, 27), with minor modifications, were used. Tissue culture wells in 96-well flat bottomed Falcon plates were incubated with gelatin (Sigma) at 30 mg/ml in water for 2 h at 37°C, aspirated to dryness and then thoroughly dried in an oven at 40°C for a further 2 h. The wells were then cooled, and 50 μl of TSP or Fn (Calbiochem) at 40 μg/ml incubated in each well for 2 h at room temperature. HBSS alone was used as a control. The protein suspension was then aspirated, the wells washed twice with HBSS, and the remaining medium was aspirated. Mature (5 to 7 d) Mϕ were released from adherent tissue culture by incubation with 5 mM EDTA in the cold, washed as above, and suspended at 10^6/ml in HBSS alone or HBSS with inhibitors/controls. The following inhibitors or controls were dissolved in HBSS and used at the stated concentration, either alone or in combination: RGDS and RGES at 2.2 mM; the CD36 mAb SMϕ, the $\alpha_v\beta_3$ mAb 23C6, and the control mAbs P112 and OX7 were all in the form of ascitic fluid diluted 1:25. From these cell suspensions, 50-μl aliquots were immediately added to protein-coated wells for 45 min at room temperature followed by washing with 0.9% saline at 4°C. The wells were fixed with 2% glutaraldehyde in PBS, and the number of adherent Mϕ in 10 randomly selected microscope fields counted using an inverted instrument with a 40× objective.

Treatment of PMNs and Mϕ with cycloheximide

PMNs isolated from the same donor were cultured either under standard conditions (see above), or in conditions that were identical, except for the presence of cycloheximide at 5×10^{-6} M. Before interaction with Mϕ under standard conditions, PMNs were washed three times to ensure removal of cycloheximide, and viability was determined by trypan blue exclusion.

Adherent Mϕ in 24-well plates were washed twice in HBSS, then incubated for 6 h in 5% CO_2 at 37°C in Iscove's DME as a control, or with the same medium containing cycloheximide at 2.5×10^{-6} M. At the end of this period, aliquots of medium were taken for measurement of TSP concentration by ELISA (see above). The wells were then washed three times and interacted with aged neutrophils or EIgG under standard conditions. In some experiments, the interaction medium contained TSP, Fn, or human albumin at 4 μg/ml.

Results

Macrophage-synthesized TSP participates in phagocytosis of apoptotic neutrophils. We first investigated whether TSP was present in the standard interaction assay in which 10^6 Mϕ were coincubated with 2.5×10^6 aged PMN in 300 μl of serum-free medium for 30 min. In keeping with previous studies (29, 33, 34), TSP could be demonstrated in two forms. First, TSP was detected in medium at the end of the interaction at a concentration of 0.59±0.14 μg/ml ($n = 6$); second, low levels of TSP were detected on the surface of Mϕ by immunofluorescence flow cytometry (Fig. 1). However, TSP could not be demonstrated by the same techniques on the surface of aged PMN (data not shown). Furthermore, we confirmed that the capac-

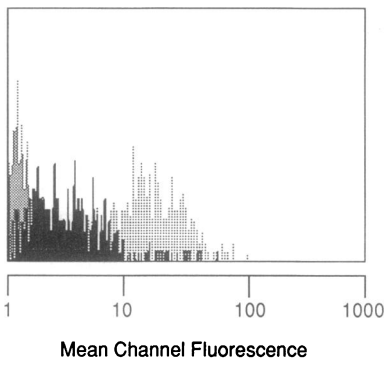

Figure 1. Expression of surface TSP by Mϕ. Typical fluorescence histograms from flow cytometry of Mϕ, showing binding of the IgG_1 anti-TSP mAb C6.7 compared with positive control CD36 mAb and negative control OX1 mAb. ■, antithrombospondin; ■, negative control OX1; ▨, CD36.

ity of Mϕ to synthesize TSP was comparable to that previously reported (29), and was over 40-fold greater than that of aged PMN (Table I). Finally, to determine if TSP might play a role in Mϕ recognition of aged PMN, we studied whether the interaction was influenced by manipulation of Mϕ TSP synthesis with 2.5 μM cycloheximide (Table II). This treatment markedly reduced the proportion of Mϕ taking up aged PMNs but did not affect uptake of EIgG. Furthermore, although cycloheximide treatment may be expected to inhibit synthesis of a wide range of proteins, "adding back" of TSP at 4 μg/ml (but not Fn or albumin) to cycloheximide-inhibited Mϕ partially "rescued" the capacity to ingest aged PMN (Table II). Aging of freshly isolated PMNs for 22 h in the presence of 5 mM cycloheximide did not inhibit their subsequent recognition by Mϕ. Thus, treated PMNs were taken up by 55.9±4.2% of Mϕ, compared with 48.1±4.7% uptake of untreated PMNs by Mϕ (n = 4). These data indicated that macrophage-synthesized TSP plays a role in recognition of aged PMN.

Exogenous TSP modulates macrophage recognition of apoptotic neutrophils. To examine whether TSP might be playing an adhesive role in the interaction, exogenous TSP was included in the interaction medium (Fig. 2). At concentrations up to a peak of 5 μg/ml, added TSP increased the proportion of Mϕ ingesting aged PMN. This effect was specific as albumin (Fig. 2), Vn and Fn (12) at the same concentrations had no effect, and the potentiating effect of added TSP was completely inhibitable by mAb against TSP (Table III). Furthermore, TSP did not induce Mϕ recognition of freshly isolated PMN. This indicated that although Mϕ have TSP associated with their surface, availability of "extra" TSP in solution appears to potentiate the interaction. Indeed, it appeared that the potentiating effect of 5 μg/ml TSP could be exerted at either cell surface, since preincubation of either cell type with TSP enhanced subsequent interaction under standard conditions (Fig. 3). By contrast, at higher concentrations, TSP specifically inhibited Mϕ recognition of aged neutrophils; albumin had no effect at 100 μg/ml, and TSP at this concentration did not affect Mϕ uptake of a control particle, the IgG-opsonized erythrocyte. Although these results suggested that the role of TSP in the interaction might be to form a "molecular bridge" between Mϕ and aged PMN, further supportive evidence would come from demonstration that (*a*) TSP can support adhesion by both cell types; and (*b*) inhibition of TSP binding to either cell type inhibits the interaction.

Table I. Release of TSP into Medium Over 6 h: Mϕ Compared with Aged PMN

Cell	Conditions	TSP release
		(μg/10^6 cells/6 h)
Mϕ	Control	1.84
Mϕ	Cycloheximide	0.14*
Aged PMN	Control	0.04*

* $P < 0.05$ compared with TSP release by Mϕ under control conditions. Data are means of six observations. Over the 6-h period in each set of conditions, each cell type remained >98% viable by trypan blue exclusion. The inhibitory effect of 2.5 μM cycloheximide indicates that the majority of TSP released under control conditions had been newly synthesized.

Table II. Effect on Aged Neutrophil Recognition of Treating Mϕ with 2.5 μM Cycloheximide for 6 h (n = 12): Partial "Rescue" by TSP

Preincubation	Protein in interaction	Aged neutrophil recognition	EIgG uptake
	(at 4 μg/ml)	*(% of control±SE)*	*(% of Mϕ)*
Control		100.0±1.26	>95%
Cycloheximide		22.2±1.05	>95%
Cycloheximide	Albumin	21.5±1.22	>95%
Cycloheximide	Fn	28.4±1.51	>95%
Cycloheximide	TSP	61.1±2.75*	>95%

In these experiments, 57.3±5.3% of Mϕ recognized aged neutrophils under control conditions. * $P < 0.05$ relative to cycloheximide-treated Mϕ.

TSP supports adhesion to apoptotic neutrophils. TSP can mediate adhesion of cells of monocyte/macrophage lineage (24, 27, 34). However, it has not been known whether TSP could support adhesion by apoptotic PMNs, which would be a

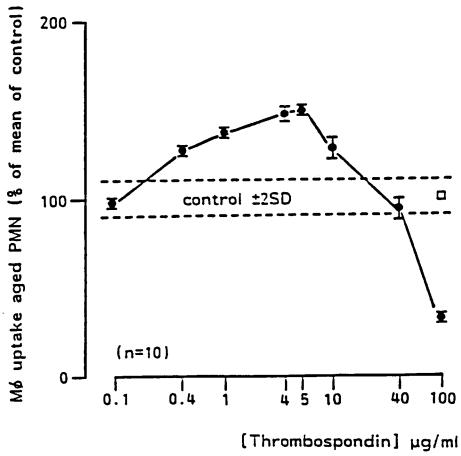

Figure 2. Effect of TSP in solution upon Mϕ recognition of aged neutrophils. Each point represents mean±SE of 10 observations made in experiments where 44.8±4.3% of Mϕ recognized aged neutrophils under control conditions. At 100 μg/ml, TSP failed to affect Mϕ uptake of EIgG (*open square*). The dotted lines represent ± 2 SD of 159 observations of Mϕ recognition of aged neutrophils made under control conditions in previous work to determine the range of intraexperimental variability of the assay (15).

Table III. Specificity of Potentiating Effect of TSP on Mϕ Recognition of Aged Neutrophils: Specific Inhibition by Anti-TSP mAb and Failure to Induce Recognition of Freshly Isolated PMN

Nature of PMN	Protein in interaction	mAb in interaction	Recognition
			(% of control±SE)
Aged	TSP 5 μg/ml	None	152.1±6.3
Aged	TSP 5 μg/ml	Anti-TSP	53.7±6.3*
Aged	TSP 5 μg/ml	Control	154.3±4.6
Fresh	None	None	3.4±0.3
Fresh	TSP 5 μg/ml	None	2.8±0.4

In these experiments, 39.0±3.4% (n = 9) of Mϕ recognized aged PMNs under control conditions. The anti-TSP MAb was C6.7 at a 1:25 dilution of ascites; the control mAb was OX7 at the same dilution. * $P < 0.05$; in separate experiments, this concentration of C6.7 was shown not to impair Mϕ ingestion of EIgG.

Table IV. Specific Binding of TSP-coated Beads to Aged PMN. Specific Inhibition by Anti-TSP mAbs C6.7, A6.1, and A2.5, but not by D4.6

Protein on bead	mAb present in bead-cell interaction	Beads binding per cell
		(mean±SE, n = 6)
TSP	—	3.26±0.07
TSP	C6.7 (anti-TSP)	0.43±0.08*
TSP	A6.1 (anti-TSP)	0.38±0.07*
TSP	A2.5 (anti-TSP)	0.46±0.07*
TSP	D4.6 (anti-TSP)	3.51±0.26
TSP	P112 (control)	3.19±0.23
Fn	—	0.17±0.03

Monoclonal antibodies were used as a 1:25 dilution of ascites. * $P <$ 0.05 compared with TSP bead binding to aged PMN under control conditions.

prerequisite for a possible "bridging" role for TSP in the interaction. Apoptotic PMNs adhered to latex beads coated with TSP to a greater degree than beads coated with a Fn, used as an adhesive protein control (Table IV). Furthermore, binding of apoptotic PMN to TSP-coated beads was specifically inhibited by three mAbs (C6.7, A6.1, and A2.5) against TSP, but not by a fourth TSP mAb, D4.6. These data indicate that TSP can support adhesion to apoptotic PMNs.

mAbs to TSP inhibit the interaction. Surface TSP was not detected on aged PMN (see above), indicating that if TSP were to "bridge" macrophage to apoptotic neutrophil, then either TSP in solution or TSP attached to the Mϕ surface must bind to the apoptotic neutrophil. Therefore, in the absence of added TSP, mAbs to TSP shown to inhibit TSP binding to apoptotic PMNs might be expected to inhibit the interaction. Specific inhibition of Mϕ ingestion of aged PMN was observed for the three mAbs, C6.7, A6.1, and A2.5, while mAb D4.6 affected neither bead binding nor the interaction (Fig. 4). No mAb from this panel inhibited Mϕ ingestion of EIgG. Furthermore, mAb 3E3, which recognizes the cell-binding domain of Fn and inhibits Fn adhesion to fibroblasts (52), failed to inhibit the interaction (data not shown), confirming that the inhibitory effect of anti-TSP mAbs was not some general property of mAbs against adhesive proteins. Therefore, these experiments provided direct evidence in support of a "physiological" bridging role for TSP in the interaction.

Inhibition of macrophage receptors for TSP specifically impairs recognition of apoptotic neutrophils. A further strategy to examine whether TSP has a bridging role in the interaction would be to manipulate Mϕ surface adhesion receptors for TSP. Attachment of macrophages to substrates coated with ligand so that receptors become modulated to the underside of the cell is a well-established method of down-regulating ligand

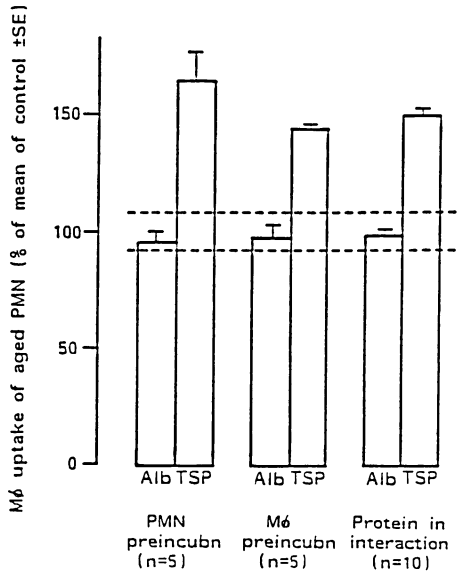

Figure 3. Localization of potentiating effect of 5 μg/ml TSP. In these experiments, where 38.1±0.7% of Mϕ recognized aged neutrophils under control conditions, aged neutrophils (*left panel*) or Mϕ (*center panel*) were preincubated with either TSP or human albumin (*Alb*), and the effects compared with presence of the proteins at the same concentration in the interaction (*right panel*). The dotted lines are ± 2 SD of control observations, as defined in Fig. 2.

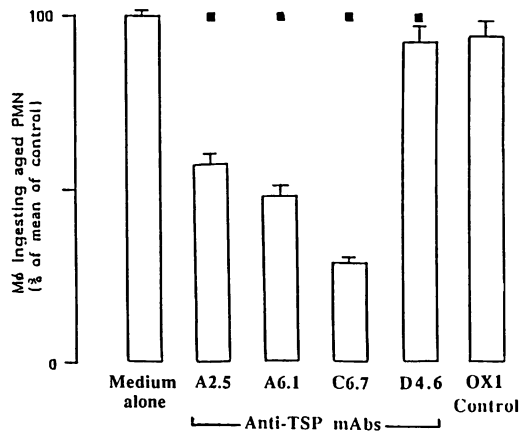

Figure 4. Monoclonal antibodies against TSP inhibit Mϕ recognition of aged neutrophils of mAbs. In these experiments, mAbs were used at a 1:25 dilution (the same concentration optimally employed in inhibition of TSP-coated latex bead adhesion to aged PMN). In this series of experiments, 37.2±2.9% of Mϕ recognized aged PMN under control conditions. No mAb affected EIgG uptake (*closed squares*).

binding at the "free" surface of the cell (49, 53). For example, we have reported that attachment of Mϕ to surfaces treated with Vn but not fibrinogen or type IV collagen inhibited subsequent recognition of aged neutrophils, in keeping with a role for Mϕ vitronectin receptor ($\alpha_v\beta_3$) in the interaction. Consequently, we examined the effect of allowing Mϕ to modulate surface receptors for TSP by attachment to tissue culture–treated surfaces coated with TSP. This maneuver resulted in strong inhibition of Mϕ phagocytosis of aged PMNs (to 19.8±3.2% of control, n = 11, in a series of experiments where 57.5±1.8% of Mϕ took up aged PMN in control conditions). There was no effect on ingestion of EIgG. In addition, treatment of the substrate with the control protein albumin had no effect. Therefore, this experiment appeared to confirm a role for TSP-binding structures on the Mϕ in recognition of aged PMNs.

This result would be expected from the reported specificity of $\alpha_v\beta_3$ for TSP (27). Nevertheless, it was also compatible with a role for other Mϕ TSP receptors, although our previous observation that fucoidan failed to inhibit the interaction indicated that involvement of Mϕ heparan sulfate proteoglycan was unlikely (26, 54). However, we had not previously sought a role for another Mϕ adhesion receptor with specificity for TSP, the 88-kD monomer, CD36 (25, 27). An IgM mAb to CD36, SMϕ (40) inhibited Mϕ recognition of aged neutrophils, but not of EIgG (Fig. 5 *A*). A number of isotype control mAbs, including mAbs binding to either cell type, had no effect (data for one mAb shown in Fig. 5 *A*), indicating that the inhibitory effect of SMϕ was highly unlikely to be caused by some nonspecific "steric" effect of IgM binding to cell surfaces. Furthermore, an IgG_1 mAb to CD36, C1Meg1 (40), also specifically inhibited phagocytosis of aged neutrophils (to 41.4±5.3% of control, n = 6; no effect on EIgG uptake was seen). The inhibitory effect of anti-CD36 mAbs was exerted at the Mϕ surface, since preincubation of Mϕ but not of neutrophils with CD36 mAbs inhibited recognition (Fig. 5 *B*) and CD36 mAbs did not bind to aged neutrophils as assessed by immunofluorescence flow cytometry (data not shown). In parallel experiments the SMϕ CD36 mAb bound specifically to Mϕ (Fig. 1). Therefore, these data indicate a hitherto unexpected role for CD36 in the interaction.

Synergistic inhibition of the interaction by mAbs to the vitronectin receptor and CD36. Previously, we reported that mAbs to both subunits of macrophage $\alpha_v\beta_3$ specifically inhibited Mϕ recognition of aged neutrophils by > 70% (12). However, in the current series of experiments a similar degree of inhibition was observed with the CD36 mAb SMϕ (Fig. 5). These observations are compatible with the possibility that Mϕ CD36 and $\alpha_v\beta_3$ function together in recognition of aged neutrophils. Thus, we studied the effects of a combination of anti-CD36 and anti-$\alpha_v\beta_3$ mAbs, each at a concentration causing only weak inhibition of the interaction when used individually. The combination exerted a strong inhibitory effect on Mϕ recognition of aged neutrophils which was synergistic rather than merely additive, in keeping with a cooperative role for the two receptors in recognition of the aged PMN (Fig. 6). This effect was specific because combination of either mAb with an irrelevant isotype control mAb did not result in synergistic inhibition and EIgG recognition was not affected.

Macrophage adherence to TSP is mediated by a "two-point" mechanism. Taken together, the data above suggest that in promoting recognition of aged neutrophils, TSP may bind to

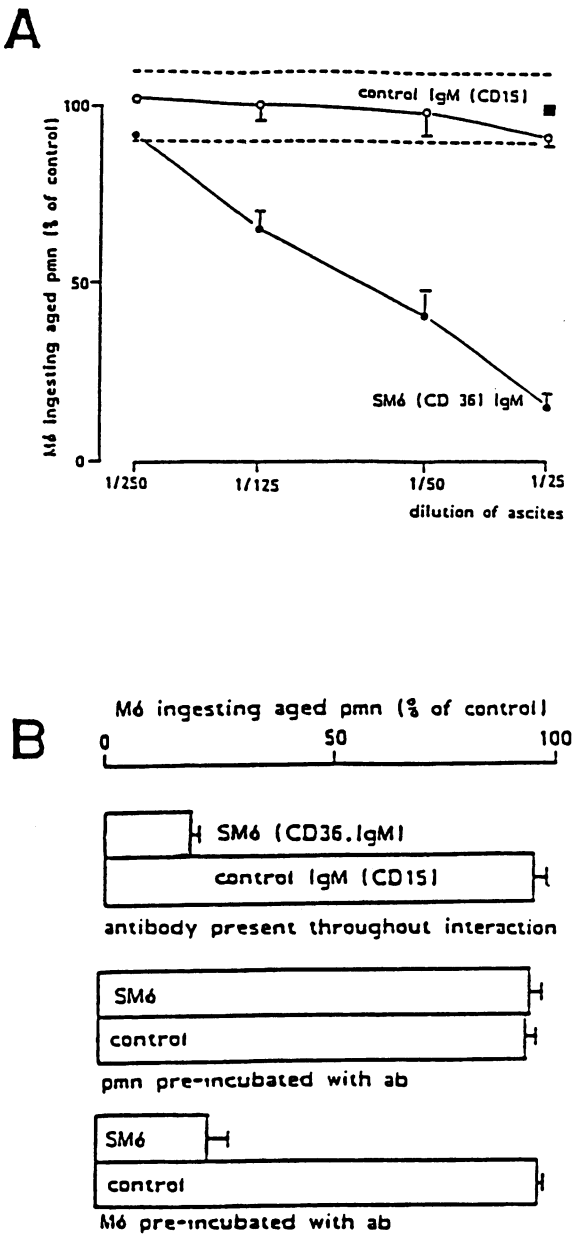

Figure 5. (*A*) Concentration-dependent inhibition by CD36 mAb SMϕ on Mϕ recognition of aged neutrophils, and (*B*) functional localization of inhibitory effect to Mϕ surface. In (*A*), each point represents the mean±SE of 12 observations made in experiments where under control conditions, 39.1±2.3% of Mϕ recognized aged neutrophils. The highest concentration of SMϕ had no effect on the proportion of Mϕ taking up EIgG (*closed square*). The control mAb was the isotype matched IgM CD15 mAb, 28. The dotted lines are as defined in Fig. 2. In (*B*), preincubation of Mϕ but not neutrophil with a 1:25 dilution of SMϕ (but not by control mAb 28) for 30 min at 4°C inhibited the interaction (*open bars:* mean±SE, n = 6) to a degree similar to that when the mAb was included only in the interaction medium. Under control conditions, 48.9±5.2% of Mϕ recognized aged neutrophils.

both $\alpha_v\beta_3$ and CD36 on the macrophage. Although inhibitor studies reported by others indicate that some cell types may use a single receptor mechanism to adhere to TSP (for example, an RGD-inhibitable integrin [27]), there are examples of cells which use a "two-point" mechanism (55). Neither the tetra-

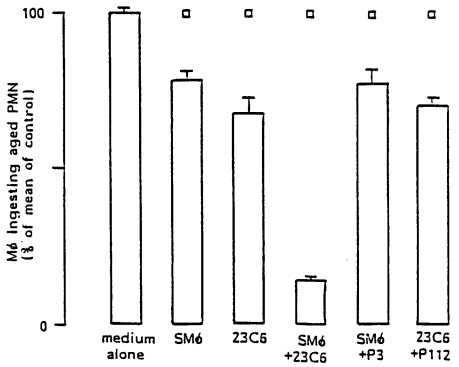

Figure 6. Synergistic inhibition of Mϕ recognition of aged neutrophils by combination of SMϕ (CD36 mAb) and 23C6 (VnR mAb) $n = 6$. SMϕ was used at a dilution of 1 in 100 ascites in HBSS, and 23C6 at 5 μg/ml in HBSS. Each mAb induced weak but specific inhibition of aged neutrophil recognition (*open bars*) and there was no effect upon Mϕ uptake of EIgG (*open squares*). "SMϕ + 23C6" signifies HBSS in which mAb SMϕ was present at a final dilution of 1 in 100 and 23C6 was also present at a final concentration of 5 μg/ml. The two right hand bars are controls for this combination with mAb P3 at a final concentration of 5 μg/ml and P112 ascites at a final dilution of 1 in 100 ascites. In this series of experiments, 64.2±1.2% of Mϕ recognized aged neutrophils under control conditions.

peptide RGDS nor the $\alpha_v\beta_3$ mAb 23C6 inhibited Mϕ binding to TSP (Table V), although both inhibit macrophage recognition of aged neutrophils. Similarly, mAb to CD36 failed to inhibit Mϕ adhesion to TSP. However, the combination of CD36 mAb with either $\alpha_v\beta_3$ mAb or RGDS (but not RGES) resulted in specific inhibition of Mϕ to TSP. The failure of these inhibitors to block Mϕ binding to Fn (56) was used as a control. These data therefore indicated that Mϕ adhesion to TSP-coated surfaces involved cooperative binding by both CD36 and the RGD-inhibitable $\alpha_v\beta_3$ vitronectin receptor integrin.

Table V. Evidence for Two-point Adhesion of Mϕ via CD36 and $\alpha_v\beta_3$ to TSP-coated Gelatin

Inhibitor(s) present in medium during adhesion assay	Macrophage binding (expressed as % of control for each adhesion)	
	TSP-coated gelatin	Fn-coated gelatin
None (Control)	100.0±2.6	100.0±1.9
SMϕ (CD36 mAb)	97.0±7.4	100.2±4.7
RGDS	95.5±4.2	105.3±14.6
RGES	99.4±6.3	91.5±6.8
23C6 ($\alpha_v\beta_3$ mAb)	86.1±6.6	96.6±1.9
RGDS + SMϕ	17.4±4.1*	98.7±8.1
RGES + SMϕ	106.6±4.2	96.1±3.6
RGDS + P112 (control mAb)	95.8±2.3	103.4±8.3
SMϕ + 23C6	14.9±1.3*	98.1±3.6
SMϕ + OX7 (control mAb)	104.7±7.3	95.8±3.7
Gelatin alone	15.5±1.0	—

Monoclonal antibodies were used as a 1:25 dilution of ascites, and tetrapeptides at 2.2 mM: When combined, the same final concentrations were achieved. In these experiments ($n = 9$), 686±51 Mϕ adhered per 10 wells to TSP-coated gelatin; 863±112 Mϕ adhered per 10 wells to Fn-coated gelatin. * $P < 0.05$ compared with TSP bead binding to aged PMN under control conditions.

Discussion

In this study, we have examined the cell surface recognition mechanisms used by human Mϕ in phagocytosis of intact aging human neutrophils that have undergone apoptosis, a potentially "injury limiting" mode of neutrophil removal operating at inflamed sites (8). Our conclusions are that macrophage-synthesized TSP mediates the interaction by forming a molecular bridge between the the cell types. Furthermore, at the Mϕ surface the data indicate that TSP binds to both CD36 and the $\alpha_v\beta_3$ "vitronectin" receptor.

There is evidence that TSP mediates platelet-platelet, platelet-erythrocyte, and monocyte-platelet adhesion by acting as a "molecular bridge" between cells (17, 21, 24, 25). These interactions may be potentiated by supplementing available TSP by addition of micromolar concentrations of exogenous TSP, and inhibited by higher concentrations which saturate TSP-binding sites. TSP-mediated agglutination of erythrocytes by activated platelets has also been reported to be inhibited by millimolar concentrations of aminosugars and basic aminoacids (16, 17). This suggests that a bridging role for TSP should be sought in Mϕ recognition of aged PMN, since we found that this interaction was also inhibited by similar concentrations of these cationic molecules (15). The data obtained in this study support the possibility that Mϕ-synthesized TSP has a bridging role in this phagocytic interaction. First, TSP is present, both in solution and associated with the Mϕ surface. Second, the interaction is inhibited by reducing supply of TSP in the interaction (by treating Mϕ with cycloheximide) and "rescued" by replenishing TSP supply. Third, the interaction is specifically potentiated by TSP, by an effect which preincubation studies show can be exerted at the surface of each cell type. Fourth, inhibition of TSP-mediated adhesion to aged PMN by mAbs to TSP inhibits the interaction. Fifth, high concentrations of soluble TSP specifically inhibit, and sixth, so does attachment of macrophages to surfaces coated with TSP, a maneuver known to down-regulate expression of adhesion receptors at the free surface of the cell. Finally, supplementation of existing TSP supply by including the protein in the interaction medium at concentrations around 5 μg/ml specifically potentiated recognition. It therefore appears reasonable to conclude that TSP plays a hitherto unexpected bridging role in Mϕ recognition of aged neutrophils. This represents an important new addition to the functional repertoire of TSP, and the first defined molecular link between the surfaces of the apoptotic PMN and the Mϕ.

Clearly, to understand the precise role played by TSP in the interaction, it will be necessary to define the receptors for TSP on both cell types. At present, the mechanisms by which TSP binds to aged neutrophils are unknown. Our current and previous data (12) indicate that CD36 and $\alpha_v\beta_3$ play their roles at the surface of the Mϕ, since in both cases the inhibitory effects of mAbs were localized to the Mϕ, and neither receptor was detectable on the surface of aged PMNs by flow cytometry. Furthermore, our previous finding that fucoidan fails to inhibit the interaction (15) suggests that neither heparan-sulfate proteoglycan nor sulfatides are involved (23, 26, 54). Since freshly isolated PMNs are not recognized by Mϕ (8), it appears likely that the mechanisms by which TSP binds to fresh PMN (31, 32) will prove to be different from those involved in TSP binding to aged PMN. Indeed, it is of interest that we found that the anti-TSP mAb A2.5 inhibited the adherence of apoptotic PMN to TSP, while others found no effect upon the adherence of

freshly isolated PMN to TSP (32). Ongoing work on the alterations occurring in the PMN surface during apoptosis will examine possible changes in the nature of the adhesive interaction with TSP.

However, the current study advances understanding of the molecular interactions occurring at the Mϕ surface during phagocytosis of apoptotic cells. First, the inhibitory effects of monoclonal antibodies to CD36 indicate a newly-identified role in recognition of aged PMNs for this 88-kD Mϕ surface molecule, the physiological function of which has been obscure. The degree of inhibition observed (> 80%) is comparable to that reported earlier for mAbs to $\alpha_v\beta_3$ (12). This suggests that the two receptors work in concert rather than mediating two independent phagocytic pathways, and this possibility was supported by synergistic inhibition of the interaction by combination of low concentrations of mAbs to CD36 and $\alpha_v\beta_3$. Second, although there has been controversy as to whether either CD36 or $\alpha_v\beta_3$ truly represent cellular adhesion receptors for TSP (57–59), our studies of Mϕ binding to TSP indicate that the two structures participate in a "two-point" adhesion mechanism for TSP. This combination of TSP receptors is different from those previously reported to mediate two-point adhesion to TSP by other cell types, but is consistent with the principle established by these workers (55). Furthermore, interference with two-point Mϕ adhesion to TSP provides an explanation for the individual and synergistic inhibitory effects of mAbs to CD36 and $\alpha_v\beta_3$ upon aged PMN ingestion. However, the data appear to raise an interesting paradox. Whereas inhibition of aged PMN ingestion occurs after blockade of either Mϕ receptor for TSP, such blockade does not inhibit Mϕ adhesion to TSP, which requires simultaneous blockade of both CD36 and $\alpha_v\beta_3$. A possible explanation is that the conformation of substrate-bound TSP, as well as the substrate itself, may allow more avid binding of Mϕ than the circumstances encountered in TSP-mediated "bridging" to the apoptotic PMN. The result would be that disruption of one receptor class is insufficient to promote Mϕ displacement from a TSP-coated substrate, while it is adequate to prevent TSP-mediated phagocytosis of aged PMN. It can be further speculated that a specific conformation of TSP presented by Mϕ CD36 and $\alpha_v\beta_3$ is required for recognition and ingestion of apoptotic PMN. The candidate TSP epitopes binding to $\alpha_v\beta_3$ and CD36 are thought to be located close to the carboxyl terminus of each subunit of the trimeric molecule (19, 20, 22), offering possibilities of dual receptor interaction with a single TSP molecule. Further study will be needed to define the stochiometry and precise molecular mechanisms of interactions between $\alpha_v\beta_3$, CD36, and TSP occurring at the Mϕ surface during recognition of apoptotic PMNs.

It is important to consider how the proposed recognition mechanism might operate at the inflamed site in vivo. The potentiating effect of low concentrations of TSP suggests that supply of this protein may be at a premium in our standard "no added protein" interaction assay, but there is evidence that TSP is in plentiful supply soon after injury of a tissue (35), presumably being secreted by platelets and a range of other cell types. Indeed, the transient presence of the molecule in the extracellular matrix of the healing skin wound corresponds with the time PMNs are being removed from this and other sites of self-limited inflammation (60). Although the $\alpha_v\beta_3$ integrin was originally thought to be specific for vitronectin (13, 61), this receptor has specificity for RGD-bearing proteins in addition to TSP, namely Fn and fibrinogen (27, 62–65). Fn has potential as a candidate for bridging aged PMN to Mϕ, since it may mediate agglutination of trypsinized erythrocytes which can be inhibited by aminosugars and basic aminoacids, and because both macrophages and neutrophils may synthesize the protein (66). However, by contrast with TSP, Fn did not enhance recognition of aged neutrophils by normal (12) or cycloheximide-inhibited Mϕ, nor did a mAb to the cell-binding domain of fibronectin inhibit recognition. Similarly, neither Vn nor fibrinogen potentiated the interaction (12). Therefore, the in vitro data mitigate against a bridging role for ligands of the $\alpha_v\beta_3$ other than TSP, and such a role also seems unlikely on the grounds that these molecules do not interact with CD36 (28, 57). However, the data suggest the speculative possibility that in addition to local changes in pH, or in concentrations of charged molecules (15), soluble or matrix-bound Fn or Vn, or proteolytic fragments of these molecules, may, in certain circumstances, inhibit aged PMN removal by interfering with TSP binding to Mϕ $\alpha_v\beta_3$, thus favouring release of PMN contents from dying cells and persistence of tissue injury.

To conclude, human monocyte-derived macrophage recognition of aging neutrophils undergoing apoptosis involves hitherto unknown functions for TSP and the macrophage surface molecule CD36. The data in this report indicate that TSP acts as a molecular bridge between apoptotic neutrophil and macrophage. At the macrophage surface, the evidence suggests that TSP forms an adhesive complex involving macrophage CD36 and the $\alpha_v\beta_3$ integrin. Further study of the mechanisms by which TSP binds to apoptotic neutrophils will provide a new approach to the identification of the surface changes that occur in apoptotic cells to lead to their recognition as "senescent-self." This study also implies that proteins that are frequently incorporated into the extracellular matrix at inflamed sites may play an important role in regulation of macrophage recognition of apoptotic neutrophils, a cellular interaction that has potential to limit the toxic potential of neutrophils in inflammation.

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